REMARKS

Claims 39-50 and 54-58 are pending in the subject application. By this amendment, Claim 39 has been amended. Applicants maintain that the claim amendment does not raise an issue of new matter. Support for the amendment can be found in the application at least on page 19, line 26 through page 20, line 10. The amendment places the application in condition for allowance or in better form for appeal. Accordingly, entry of the amendment is respectfully requested.

Rejection under 35 U.S.C. §112, First Paragraph

Claims 39-50 and 54-58 stand rejected under 35 USC §112, first paragraph, as not enabled. The Examiner stated that the specification does not reasonably provide enablement for GBSSI genetic constructs and methods of expressing and altering the affinity for starch in plants transformed therewith.

Applicants respectfully traverse this rejection.

As applicants previously noted, Flipse et al. (Theoretical and Applied Genetics 88: 369-375, 1994) clearly show in Table 1 of their publication that plants which after transformation show increased GBSS activity also show a dramatically increased amylose content, thus demonstrating that an increased activity of the enzyme indeed gives an altered phenotype of the starch.

In the current Office Action, the Examiner notes that over-expression of GSBB in Flipes et al. did not recover levels of amylase greater than that of wild type, and thus, according to the Examiner, the present invention is not enabled. In this regard, applicants respectfully point out that Flipse et al. only transformed mutant strains and not wild-type plants. Thus, a conclusion that the contents and properties of the starch of wild-type plants would not have been changed when transformed should not be made on basis of Flipse et al. Since Flipse et al. clearly show a change in the starch properties of the plants that have been transformed with the construct, Flipse et al. support the enablement of the present invention.

Further proof of the enablement of the present invention can be found in a recently published PhD thesis of G. Kok-Jacon, in which experiments have been described showing that a mutansucrase fused to a starch binding domain (SBD) was able to alter some of the characteristics (e.g. melting temperature) of starch granules. A copy of said chapter from this PhD thesis is enclosed. In short, Kok-Jacon made constructs with a truncated mutansucrase linked with the N-terminus or C-terminus of a SBD from the CGTase gene of *Bacillus circulans* strain 251 and in frame with a plastidic protein targeting sequence. These constructs were transformed into potato cv. Kardal. It was found that the constructs in which a SBD was fused to the N-terminus of the (truncated) enzyme were most effective in targeting the enzyme to the starch granules, and thus affecting changes in the starch. This publication thus clearly shows effects of constructs according to the invention in wild-type plants.

In regard to whether "undue experimentation" is required to practice the claimed invention, applicants note that the MPEP states that "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation." (MPEP §2164.01.) Applicants further note that in the field of plant genetic engineering, it is known by the person having ordinary skill in the art that normally a series of plants has to be transformed, which series will show expression of the transformed gene(s) to a varying degree. Transformants that exhibit the desired result can be readily identified on the basis of standard molecular biological techniques (such as PCR and blotting techniques). Thus, it is within the normal routine of the average person skilled in the art to perform these transformations and select the ones that are show to be successful.

Reconsideration and withdrawal of this rejection are respectfully requested.

Rejections under 35 USC §102(b)

1. Claim 39 and dependent Claims 41 and 44 stand rejected under 35 USC §102(b) as anticipated by Dalmia et al., Biotechnology and Bioengineering, 47: 575-584, 1995, in light of GenBank Accession gi: 143654, April 26, 1993.

In the present Office Action, the Examiner indicated that the claims do not set forth requirements for what is suitable for transforming a plant, so as to distinguish the claimed invention from the cited art.

In reply applicants have herein above amended independent Claim 39 to recite:

> A genetic construct comprising (a) a first nucleotide sequence encoding an enzyme that interacts with starch or starch granules, (b) a second nucleotide sequence encoding a bacterial starch binding domain, and (c) a promoter for expression in a plant of a fusion protein comprising the enzyme and the bacterial starch binding domain, wherein the construct is suitable for transforming a plant, and wherein the plant transformed with the construct expresses a fusion protein comprising the enzyme and the bacterial starch binding domain.

Applicants maintain that Dalmia et al. do not teach all the limitations set forth in Claim 39 and thus Dalmia et al. do not anticipate the claimed invention. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

2. Claims 39, 42-50, and 54-58 stand rejected under 35 USC §102(b) as anticipated by Kortstee et al., The Plant Journal 10: 83-90, 1996.

In the current Office Action, the Examiner maintains that bacterial starch binding domains are inherent to bacterial enzymes that make or break the covalent bonds of starch such as the starch branching enzyme from *E. coli* taught by Korstee encoded by *glgB*.

Applicants respectfully traverse this rejection.

Applicants submit that *glgB* does not contain a starch binding domain. In *E. coli* the enzyme catalyzes the branching in glycogen and it is active in the cytoplasma of the

cell. This is in contrast to bacterial enzymes that contain a SBD, where the enzyme reacts with starch and where the starch is located in starch granules in the cell. Such enzymes and their bacterial sources are mentioned in the specification (see the paragraph bridging pages 12 and 13). Further, a recent review on microbial starch binding domains (Rodriguez-Sanoja, R. et al., Curr. Opin. Microbiol. 8:260-267, 2005, copy enclosed) teaches that SBDs are only found in filamentous fungi, Gram-positive bacteria, Proteobacteria of the γ-subdivision, actinomyceres and Archaea in the following enzymes: α-amylases, β-amylases, maltotetraohydrolases, maltopentaohydrolases, maltogenic α-amylases, cyclodextrin glucanotransferase (CGTase), acarviose transferases and glucoamylases (see paragraph bridging two columns on page 261). The absence of the *E. coli glgB* from this comprehensive list indicates that it is commonly understood that *glgB* does not possess a starch binding domain. Accordingly, Kortstee et al. do not anticipate the claimed invention. Reconsideration and withdrawal of this rejection are respectfully requested.

CONCLUSIONS

In view of the amendments and remarks made hereinabove, reconsideration and withdrawal of the rejections in the November 22, 2005 Final Office Action and passage of the pending claims to allowance are respectfully requested.

No fee, other than the enclosed $120.00 fee for a one month extension of time, is deemed necessary in connection with the submission of this reply. However, if any unanticipated fee is required to maintain the pendency of the subject application, the PTO is authorized to withdraw the amount of any such fee from Deposit Account 01-1785. Please credit overpayments to Deposit Account 01-1785.

Respectfully submitted

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March 21, 2006

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Expression of different glucansucrases in potato tubers: Implications for starch biosynthesis

Géraldine Kok-Jacon

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M. J. Kropff,
in het openbaar te verdedigen
op maandag 19 september 2005
des namiddags te vier uur in de Aula.

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Granule-bound mutansucrase alters melting temperature of starch granules

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Abstract: It has been shown previously that mutan can be co-synthesized with starch when a truncated mutansucrase (GTFICAT) is directed to potato tuber amyloplasts. The mutan seemed to adhere to isolated starch granules, but it was not incorporated in the starch granule. In this study, *GtfICAT* was fused to the N- or C-terminus of a starch-binding domain (SBD). These constructs were introduced in potato plants (cv. Kardal), in order to bring GTFICAT in more intimate contact with growing starch granules, and to facilitate the incorporation of mutan polymers in starch. For the high SBD-GTFICAT expressors, it was found that mutan was present inside starch granules, demonstrating that granule-targeting of GTFICAT was successful. Interestingly, the granules of these transformants had spongious surfaces, a higher melting temperature, and a more pronounced retrogradation behaviour, compared to those from controls. Except for the T onset, these alterations were less pronounced than those observed in transformants expressing *GtfICAT* gene without appended SBD. *In vitro* production of mutan by incubating starch granules from transformants with an excess of sucrose was not evidenced. Our results show that expression of granule-bound and "soluble" GTFICAT can affect starch biosynthesis differently, and that the appended SBD inhibits the activity of GTFICAT in the engineered fusion protein.

To be submitted

Key words: mutan, glucansucrase, starch-binding domain, granule-boundness, transgenic potato

Introduction

In a previous study, we have shown that expression of the truncated mutansucrase gene *GtfICAT* (*Gtfl* without a glucan-binding domain) in potato amyloplasts led to a more efficient production of mutan in comparison to that of the full-length *Gtfl* (Kok-Jacon *et al.*, 2005a). Mutan production by GTFICAT was accompagnied with pronounced morphological and physicochemical alterations at the tuber and starch levels. However, from this study, the presence of mutan inside starch granules was not evidenced. We hypothesized that even more dramatic effects on starch granule morphology and properties might be obtained, if mutan could be incorporated in the starch granule. This might be achieved by bringing GTFICAT and the growing starch granule in intimate contact with each other. For this purpose, we have engineered two genes in which a starch-binding domain (SBD) was fused to either the 5' or the 3' end of GTFICAT. It has been shown before that SBD is an efficient tool for targeting effector proteins to the growing starch granule (Ji *et al.*, 2003; 2004b). The use of SBD technology may offer three advantages. (i) Mutan will be produced close to the granule surface, which will increase the chance that it co-crystallizes with starch during granule formation. (ii) The more intimate contact between GTFICAT and starch may facilitate the so-called acceptor reaction of the enzyme (Monchois *et al.*, 2000), which in turn may lead to covalent attachment of mutan to starch. (iii) Mutan may be produced post-harvest by supplying sucrose, a cheap and abundant substrate, to transgenic starch granules containing the fusion proteins (Kok-Jacon *et al.*, 2003).

In this study, we investigate if starch granule properties can be more severely affected with a granule-bound GTFICAT than with a "soluble" GTFICAT. For this, SBD was fused to the N- or C-terminus of GTFICAT, since our previous studies have pointed out that the position of the SBD in the fusion protein can influence the activity of the appended effector. Although previous studies (Ji *et al.*, 2003; 2004b) have shown that accumulation of SBD was most efficient in the amylose-free (*amf*) potato background, we have chosen to express the engineered genes in the Kardal genotype. In this way, a direct comparison with the GTFICAT potato transformants that had already been obtained is possible (Kok-Jacon *et al.*, 2005a). Post-harvest experiments with transgenic granules and sucrose were also performed, in order to alter the polymer composition of the granules by the *in vitro* production of mutan.

Materials and

Preparation of the N- and C-te

pPF and pPF*Gt* SBD-linker and respectively. Th plasmid (Ji *et al* originated from linker fragment (Gilkes *et al.*, 1 obtained by PC AGTACTATGG site (5'- TCGCG

A) RB

B) RB

Figure 1. Sche for potato plant

For the consti amplification GATATCTCCC (5'- CCCCGG resulting in pP *GtfICAT* fragm CCCGGGACT

Materials and methods

Preparation of constructs containing the *GtfICAT* gene and the SBD fragment cloned at the N- and C-terminal positions

pPF and pPF*GtfICAT* (Kok-Jacon *et al.*, 2005a) were used as starting material for cloning the SBD-linker and linker-SBD fragments resulting in pPF*SBDGtfICAT* and pPF*GtfICATSBD*, respectively. The SBD-linker and linker-SBD fragments were obtained from the pTrcHisB/SBD2 plasmid (Ji *et al.*, 2004a) that was used as a template for PCR amplification. The SBD fragment originated from the *CGTase* gene of *Bacillus circulans* strain 251 (Lawson *et al.*, 1994) and the linker fragment is similar to the Pro-Thr-rich linker of the *Cellulomonas fimi* exoglucanase (*Cex*) (Gilkes *et al.*, 1991). For the construction of pPF*SBDGtfICAT*, the SBD-linker fragment was obtained by PCR amplification with a forward primer, containing a *ScaI* restriction site (5'- AGTACTATGGCCGGAGATCAGGTC- 3'), and a reverse primer, containing a *NruI* restriction site (5'- TCGCGACGACGGGGTC- 3'), and cloned into the *SmaI* restriction site of pPF*GtfICAT*.

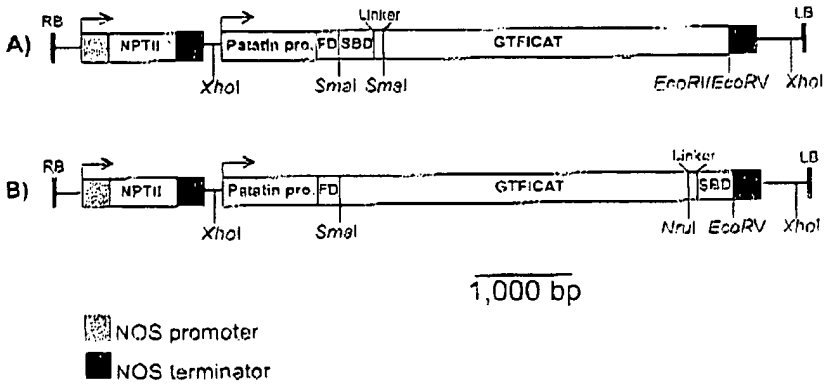

Figure 1. Schematic representation of pPFSBDIC (**A**) and pPFICSBD (**B**) binary vectors used for potato plant transformation.

For the construction of pPF*GtfICATSBD*, the linker-SBD fragment was obtained by PCR amplification with a forward primer, containing an *EcoRV* restriction site (5'- GATATCTCCGACGCCGACGC- 3'), and a reverse primer, containing a *SmaI* restriction site (5'- CCCCGGGATCCACCAAAAC- 3'), and cloned into the *EcoRV* restriction site of pPF resulting in pPFSBD. In order to remove the stop codon in the original *GtfICAT* sequence, the *GtfICAT* fragment was amplified with a forward primer, containing a *SmaI* restriction site (5'- CCCGGGACTGAAAACTGTTAG- 3'), and a reverse primer, containing a *NruI* restriction site

(5'- TCGCGAACATTGAGGTACTTG- 3'), and cloned into the *Sma*I restriction site of pPFSBD, resulting in pPF*GtflCATSBD*. pPF*SBDGtflCAT* and pPF*GtflCATSBD* were completely sequenced in one direction by Baseclear (The Netherlands) to verify the correctness of the construct. pPF*SBDGtflCAT* and pPF*GtflCATSBD* were digested with *Xho*I and ligated into a pBIN20 binary vector (Hennegan and Danna, 1998), resulting in pPFSBDIC and pPFICSBD (Fig. 1).

Transformation and regeneration of potato plants

pPFSBDIC and pPFICSBD were transformed into *Agrobacterium tumefaciens* strain LBA 4404 using electroporation (Takken *et al.*, 2000). Internodal stem segments from the tetraploid potato genotype (cv. Kardal (KD)) were used for *Agrobacterium*-mediated transformation which was performed as described by Kok-Jacon *et al.* (2005b).

Starch isolation

Starch isolation was performed as described by Kok-Jacon *et al.* (2005a).

Expression analysis of *GtflCAT* and genes involved in starch synthesis, using semi-quantitative and real-time quantitative RT-PCR analysis

RNA was isolated from 3 g (fresh weight) of potato tuber material from selected transgenic lines according to Kuipers *et al.* (1994).

Semi-quantitative and real-time quantitative RT-PCR were performed as described by Kok-Jacon *et al.* (2005b). *GtflRT* primers, 5'-CCGTGCTTACAGTACCTCAGC-3' and 5'-GGTCGTTAGCATTGTAGGTGAAA-3' (Tm=59°C, 35 cycles) were based on the *Gtfl* gene sequence (Ferretti *et al.*, 1987). RNA sample from the RVT34-77 transformant was used as described by Kok-Jacon *et al.* (2005a).

Determination of morphological and physicochemical properties of starch granules

Analysis of starch granule morphology was performed by light microscopy (LM) and scanning electron microscopy (SEM) as described by Kok-Jacon *et al.* (2005b). Mutan polymers were visualized with LM and the exo-mutanase treatment was performed as described by Kok-Jacon *et al.* (2005a). Median values of the granule size distribution (d_{50}), gelatinization analyses, amylose content, starch content, chain length distributions (HPSEC, HPAEC) were determined as described by Kok-Jacon *et al.* (2005b), and viscosity profiles as described by Kok-Jacon *et al.* (2005a).

Determination of SBD content of transgenic starches by dot blot analysis

Dot blot analysis was performed according to the method described by Ji *et al.* (2003). A 12.5 % sodium dodecylsulphate-polyacrylamide gel (50 mm × 50 mm × 3 mm), with nine equally spaced holes (9 mm diameter), was placed in contact with a similarly sized Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK). Twenty milligrams of (transgenic) starch was boiled for 5 min with 200 µl of 2 × SDS sample buffer (Murashige and Skoog, 1962). After cooling to room temperature, the starch gel was transferred into one of the holes. SBD proteins from transgenic starch gels were blotted onto the membrane with PhastSystem (Pharmacia, Sweden; 20 V, 25 mA, 15°C, 45 min). The blot was incubated overnight in a 1 % blocking solution (10 ml 10 × western blocking reagent; Roche, Germany) in 90 ml TBS (20 mM Tris, 500 mM NaCl pH 7.5) at room temperature. Subsequently, the blot was washed in TTBS (0.05 % Tween-20 in TBS) for 5 min, and incubated for 2 h at room temperature with a 1:500 dilution of the primary antibody (antiSBD) in a 0.96 % blocking solution in TTBS. After this, the blot was washed twice in TTBS for 5 min, and incubated for 1 h at room temperature with a 1:2000 dilution of Goat Anti-Rabbit IgG (H + L) Alkaline Phosphatase Conjugate (BioRad, USA) in a 0.64 % blocking solution in TTBS. The blot was washed twice in TTBS, and once in TBS for 5 min. Finally, the blot was stained with a 0.1 M $NaHCO_3$ solution pH 9.8 containing 1 % NBT/BCIP (Roche Molecular Biochemicals, Germany), and 0.01 M $MgCl_2$.

Post-harvest experiments

Post-harvest experiments were performed using 10 mg of starch, which was incubated for 66 h in 1 ml of 50 mM Tris/HCl pH 7.0 and 1 M sucrose at 37°C and 45°C. After centrifugation (1 min; 10,000 g), the supernatant was submitted to HPAEC analysis.

HPAEC was performed as described previously except that the column temperature was 28°C and a different gradient was used. Three eluents were used, eluent A (100 mM NaOH), eluent B (1 M NaAc in 100 mM NaOH) and eluent C (H_2O) as follows: 0→ 12 min (linear gradient 25 to 85 % eluent A; 75 to 15 % eluent C); 12→ 25 min (linear gradient 0 to 10 % eluent B; 15 to 5 % eluent C); 25 → 25.1 min (linear gradient 85 to 0 % eluent A; 10 to 100 % eluent B; 5 to 0 % eluent C); 25.1 min→ 30 min (100 % eluent B; rinsing phase); 30→ 45 min (0 to 25 % eluent A; 100 to 0 % eluent B; 0 to 75 % eluent C; equilibration). The eluents were monitored by an ED40 electrochemical detector in the pulsed amperometric mode (Dionex).

Results

Expression of SBD-GtfICAT results in altered tuber phenotype

The SBD-GtfICAT and GtfICAT-SBD fragments were cloned in frame to the plastidic protein targeting sequence (FD) (Gerrits *et al.*, 2001), that was driven by the highly tuber-expressed patatin promoter (Wenzler *et al.*, 1989) resulting in the pPFSBDIC and pPFICSBD constructs (Fig. 1). These constructs were used for *Agrobacterium*-mediated transformation of potato cv. Kardal (KD) from which thirty independent transgenic potato clones were obtained that were named KDSICxx and KDICSxx, respectively. SIC and ICS represent the SBD-GtfICAT and GtfICAT-SBD genes, respectively, and xx the clone number. The untransformed genotype is referred to as KD-UT. During growth, the transgenic plants were morphological similar to the controls (data not shown).

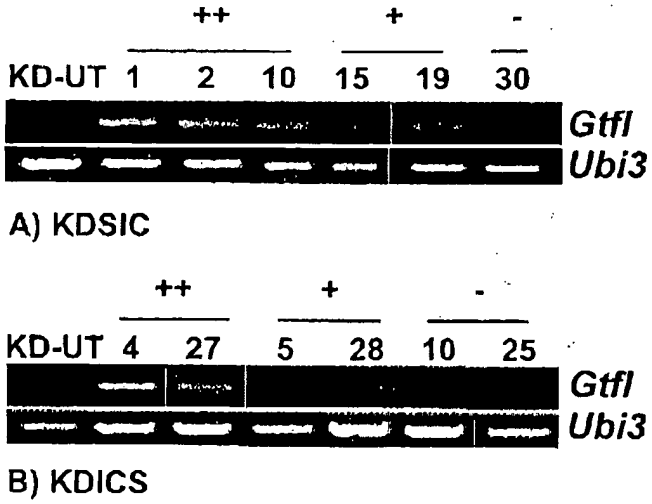

Figure 2. RT-PCR analysis of the selected KDSIC and KDICS transformants, and KD-UT. The upper panel shows the PCR products using the primers designed on the *Gtfl* sequence and the lower panel, the PCR products using the primers designed on the *Ubi3* sequence that served as an internal control.

GtfICAT expression was monitored by RT-PCR analysis, from which a number of transformants were selected and divided in classes, based on the band intensity of the PCR products. In parallel, the ubiquitin-ribosomal gene expression (*Ubi3*), which is known as constitutive (Garbarino and Belknap, 1994), was used as an internal control. The (–), (+) and (++) classes were defined as no, intermediate and high levels of

mRNA, respectively. From the KDSIC series, the KDSIC30 (–), KDSIC15 (+), KDSIC19 (+), KDSIC1 (++), KDSIC2 (++) and KDSIC10 (++) transformants were selected for further characterization (See Fig. 2A). In addition, transformants from the KDICS series such as KDICS10 (–), KDICS25 (–), KDICS5 (+), KDICS28 (+), KDICS4 (++) and KDICS27 (++), were also selected (See Fig. 2B). Due to the low *Ubi3* intensity of KDICS5, this transformant could be also assigned to the (++) class. As shown in Figure 2, no *GtfICAT* mRNA was detected in the KD-UT plants.

Tuber phenotype of the (++) (Fig. 3) and (+) classes (data not shown) of the KDSIC series was severely affected, showing brownish/reddish discolourations. Concerning the (–) class of transformants, the tuber phenotype was comparable to that of KD-UT. No phenotypical differences were observed for tubers of the KDICS series, which was comparable to KD-UT. In general, tuber number and yield were unchanged for the KDSIC and KDICS series, and were similar to KD-UT.

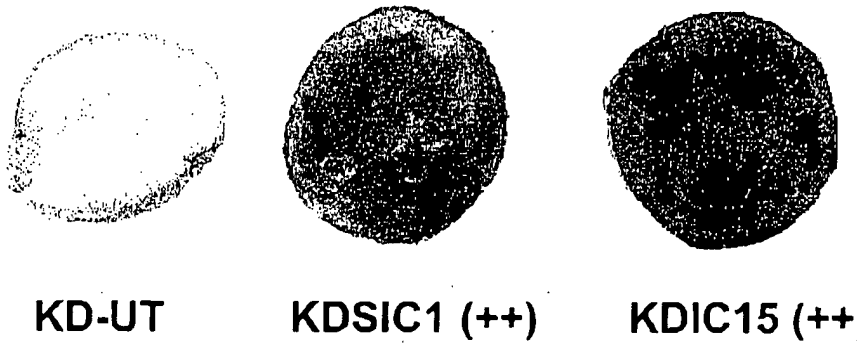

Figure 3. Tuber phenotype of KDSIC1 (++) and KDIC15 (++) transformants compared to that of KD-UT.

Accumulation of SBD-containing protein in the KDSIC10 transformant

Accumulation of SBD-containing protein in the starch granules was monitored by Western dot blot analysis, using gelatinized starches from all the selected transformants. Determination of SBD protein concentration was performed by using calibrated positive controls from the (+), (++) and (+++) classes of the *amf*S series of transformants described by Ji et *al.* (2003). Subsequently, the dot intensities from all the gelatinized starches were compared to these positive controls. The KDSIC10 transformant exhibited a dot intensity comparable to that of the (+) class (data not

shown), showing that SBD proteins accumulated inside or at the starch granule surfaces. Concerning the other selected transformants, the dot intensity was comparable to that of KD-UT, in which accumulation of SBD proteins was too low to be detected or absent (data not shown).

Detection of mutan in only the (++) and (+) classes of KDSIC transformants

As described previously (Kok-Jacon *et al.*, 2005a), mutan can be visualized using an erythrosine red dye (Fig. 4). This staining was also used on a positive control,

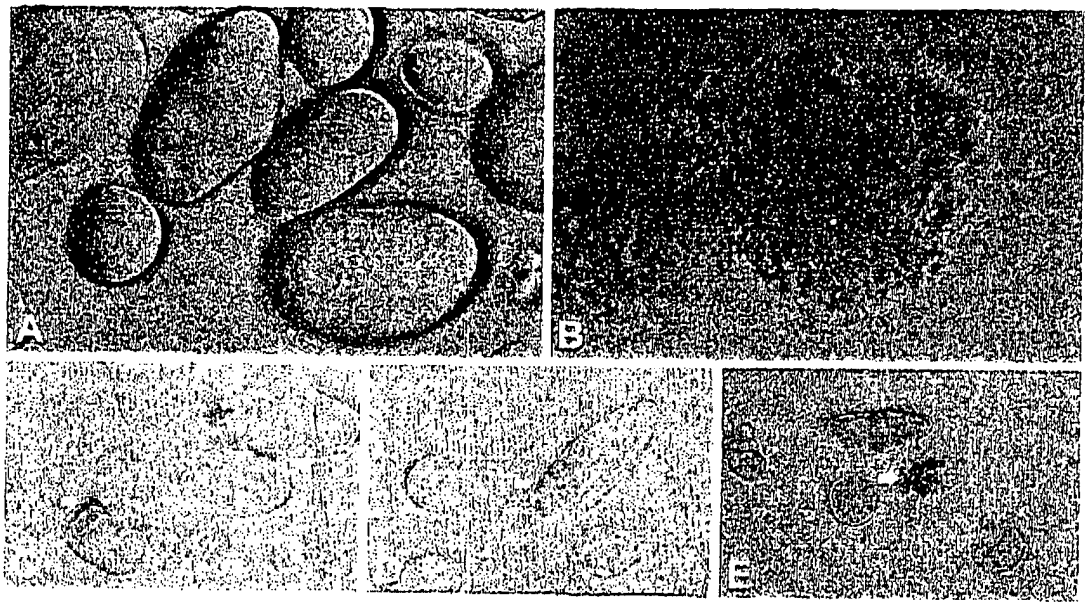

Figure 4. LM analysis of starch granules (x 800) stained with Disclosing solution red-Cote from KD-UT (**A**) and KDSIC10 (**C**) compared to that of mutan (**B**). Treatment of KDSIC10 (**D**) and KDIC15 (E) starch granules with 25 mU of mutanase enzyme.

consisting of purified mutan (Wiater *et al.*, 1999) (Fig. 4B). A number of starch granule surfaces from the (++) class, illustrated in Figure 4C, and (+) classes (data not shown) of the KDSIC series coloured red, which demonstrated the presence of mutan. Starch granules from the (–) class transformants did not stain with the erythrosine dye (data not shown) similar to those of KD-UT (Fig. 4A). For none of the KDICS starch granules, a red colouration was observed upon erythrosine treatment (data not shown). KDSIC10 starch granules were exhaustively treated with an exo-mutanase solution in order to

detach the mutan from the granule surface. From previous results (Kok-Jacon *et al.*, 2005a), it was shown that the 48 h incubation time was sufficient for detaching mutan from the granule surfaces (Fig. 4E). From Figure 4D, it can be seen that most of the mutan remained attached to the granule surface, which suggests that a proportion of the mutan is present inside the starch granules, and therefore inaccessible to the exo-mutanase.

Altered granule phenotype in the KDSIC series

SEM analysis was performed on the selected transgenic starches. With SEM, the presence of altered starch granules was visualized in the (++) class of the KDSIC series (Fig. 5: B-F-G-H) in contrast to the (–) class (data not shown), which was comparable to that of KD-UT (Fig. 5A). From Figure 5 (F, G and H), it can be seen that surfaces of starch granules were spongy and irregular. In addition, small protrusions on the starch granule were also present. Such granule phenotypes were not observed in the (++) class of the KDIC transformants (Fig. 5D and I) demonstrating that N-terminal

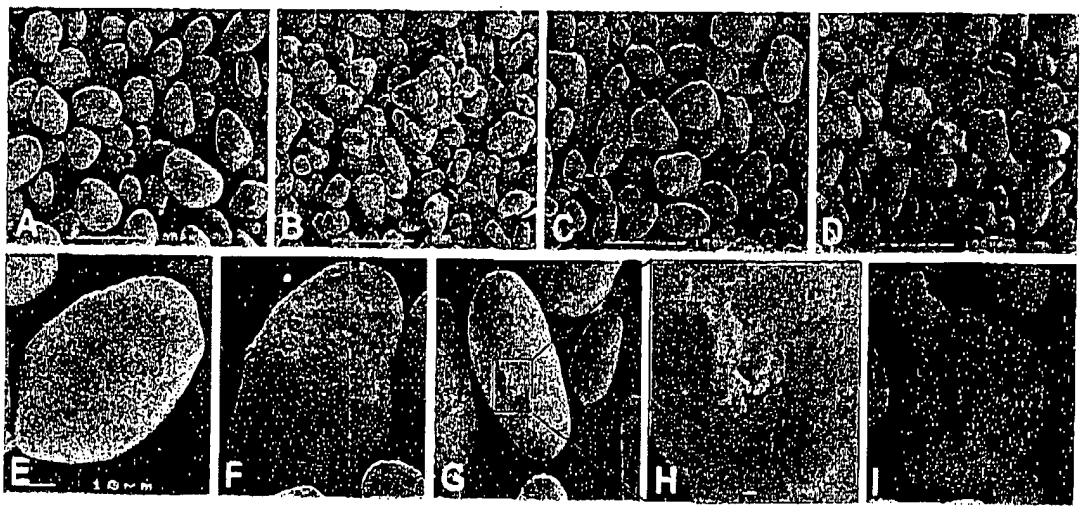

Figure 5. SEM analysis of starch granules (x 350) from KD-UT (**A**) compared to that of selected transformants (KDSIC10 (**B**), KDICS27 (**C**), KDIC15 (D)) and examples of starch granules (x 1,000) with altered morphology (KDSIC10 (**F**), KDSIC10 (**G**), KDIC15 (I) and KDSIC10 (x 5,000) (H)) compared to KD-UT (E).

fusion of SBD to the *GtfICAT* gene interferes differently with starch granule morphology. For the (++) class of the KDICS series (Fig. 5C), starch granules were not

significantly altered, and similar to those of KD-UT. From the SEM pictures, the number of altered granules was scored by counting a population of 100 starch granules in triplicate for one transformant of the (–) and two transformants of the (++) class of each series. This quantification is shown in Figure 6 for the KDSIC30 (–), KDSIC1 (++), KDSIC10 (++) and KDICS25 (–), KDICS4 (++), KDICS27 (++) transformants. The

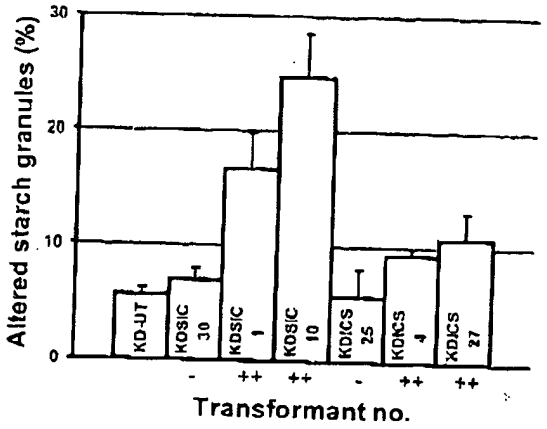

Figure 6. Percentage of starch granules with altered morphology comparable to those of Figure 5 (F, G and H) in selected transformants and KD-UT plants.

highest number of altered starch granules was found in the (++) class of the KDSIC series, ranging from 16.7 ± 3.1 for KDSIC1 to 24.7 ± 3.8 for KDSIC10. In the (++) class of the KDICS series, the number of altered starch granules was similar as in KD-UT (5.7 ± 0.6), ranging from 9.3 ± 0.6 for KDICS4 to 10.7 ± 2.3 for KDICS27. For the (–) class of transformants, the frequency of altered starch granules was about 7 %. In general, it seems that the SBD-GTFICAT fusion protein affects the starch granule phenotype more dramatically than GTFICAT-SBD. However, in the first case, the starch granule phenotype was less affected than that of (++) KDIC transformants in which 31.3 ± 2.3 % of the starch granules exhibited altered morphologies (Kok-Jacon *et al.*, 2005a).

SBD-GTFICAT expression alters viscosity profile

Median granule size (d_{50}), amylose and starch contents of the transgenic starches were determined, from which no significant differences were found in comparison to KD-UT (Table 1). A direct correlation between tuber browning and decreased starch content

Table 1: Summary of SBD expression (classification according to *Ji et al.*, 2003), granule size (d_{50}: median value of the granule size distribution), gelatinization characteristics (T_o = T onset: temperature of onset of starch gelatinization), amylose and starch contents measurements of starches from the KDSIC10 (++), KDICS27 (++), KDIC15 (++; Kok-Jacon *et al.*, 2005a) transformants and KD-UT. Data (± SD) are the average of two or three independent measurements.

Transformants	SBD expression	d_{50} (µm)	T_o (°C)	Amylose content (%)	Starch content (mg/g FW)
KD-UT	–	28.8 (± 0.5)	66.7 (± 0.1)	20.1 (± 0.5)	242.9 (± 93.5)
KDSIC10 (++)	+	25.1 (± 0.8)	69.5 (± 0.1)	18.0 (± 0.2)	179.7 (± 38.3)
KDICS27 (++)	–	28.6 (± 0.2)	67.4 (± 0.1)	20.0 (± 0.2)	191.6 (± 29.4)
KDIC15 (++)	–	20.2 (± 0.4)	67.2 (± 0.1)	19.7 (± 0.2)	81.3 (± 37.3)

could not be found, in contrast to transformants only expressing GTFICAT (Kok-Jacon *et al.*, 2005a). Changes in the temperature of onset of starch gelatinization (T onset (T_o)) were only detected for the KDSIC10 transformant (Table 1), exhibiting a T onset of 69.5°C which is about 3°C higher than that of KD-UT (66.7°C). Viscosimetric analysis showed that the KDSIC10 profile (Fig. 7 (2)) was different from that of KDICS27 (Fig. 7

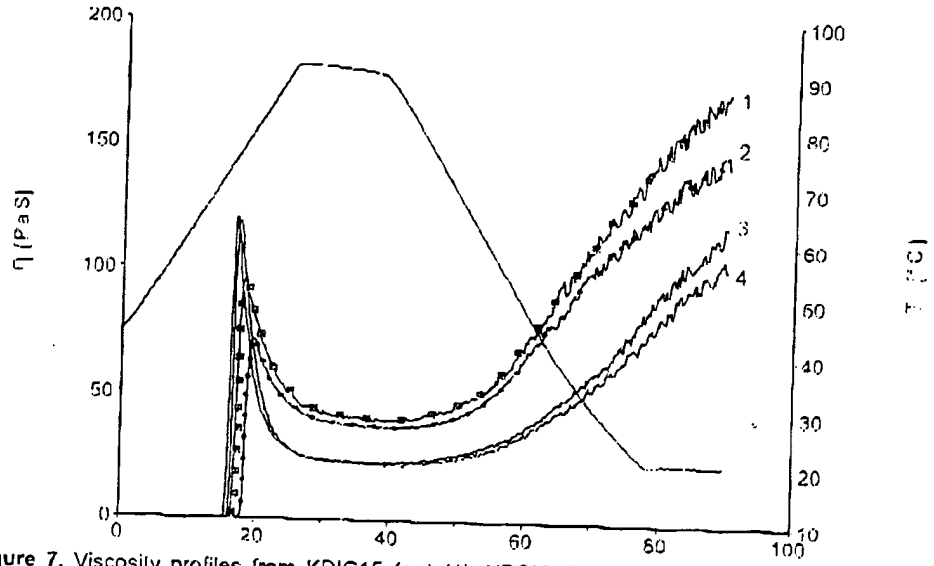

Figure 7. Viscosity profiles from KDIC15 (++) (1), KDSIC10 (++) (2) and KDICS27 (++) (3) transformants compared to that of KD-UT (4).

(3)) and KD-UT (Fig. 7 (4)). The T onset was about 3°C higher than that of KD-UT and KDICS27, which is in accordance with the DSC results. In addition, the end viscosity

was about 1.5-fold higher in comparison to KD-UT and KDICS27, but not as high as that of KDIC15 (Fig. 7 (1)). These results illustrate that N-terminal fusion of SBD to *GtfICAT* induced more severe effects in the physicochemical properties of the starch compared to the C-terminal SBD fusion, but not as pronounced as GTFICAT alone. It should be noted that some variation in the values for peak viscosity was observed, in contrast to those for end viscosities, which were very consistent. Within the KDSIC series, also the starches from KDSIC30 (-), KDSIC1 (++), and KDSIC2 (++) were subjected to viscosimetric analysis. KDSIC30 starch showed a behaviour similar to KD-UT starch, whereas KDSIC1 and KDSIC2 starch gave a pattern intermediate between KDSIC30 and KDSIC10. This is consistent with the results from RT-PCR and Western blot analysis.

GTFICAT with appended SBD does not seem to alter starch fine structure

The chain length distribution was determined in order to detect deviations in starch structure, which may indicate the presence of α-(1→3)-linked glucose residues. After complete debranching of starch with isoamylase, no consistent changes were detected with HPSEC and HPAEC in comparison to KD-UT starches (data not shown). In addition, isoamylase debranched starches were further incubated with α-amylase and analyzed with HPAEC in order to detect the presence of possible α-(1→3) linkages. Despite the pronounced alterations of the rheological properties, in particular for KDSIC10, no consistent changes in amylopectin fine structure were detected in comparison to KD-UT starches (data not shown). These results suggest that mutan is not covalently attached to the starch polymers, but rather is present as a separate carbohydrate.

Expression levels of *AGPase* and *GBSSI* genes are down-regulated in the (++) KDSIC class and that of *SuSy* in the (++) KDICS class

As described previously (Chapters 2, 3 and 4), key enzymes involved in starch biosynthesis such as sucrose synthase (SuSy), ADP-glucose pyrophosphorylase (AGPase), starch synthase III (SSIII), starch branching enzyme I (SBEI) and granule-bound starch synthase I (GBSSI) were selected and their expression levels were monitored by real-time quantitative RT-PCR (Fig. 8). In the KDSIC series, the expression levels of *AGPase* and *GBSSI* genes were down-regulated in the (++) class

Expression relative to *Ubi3*

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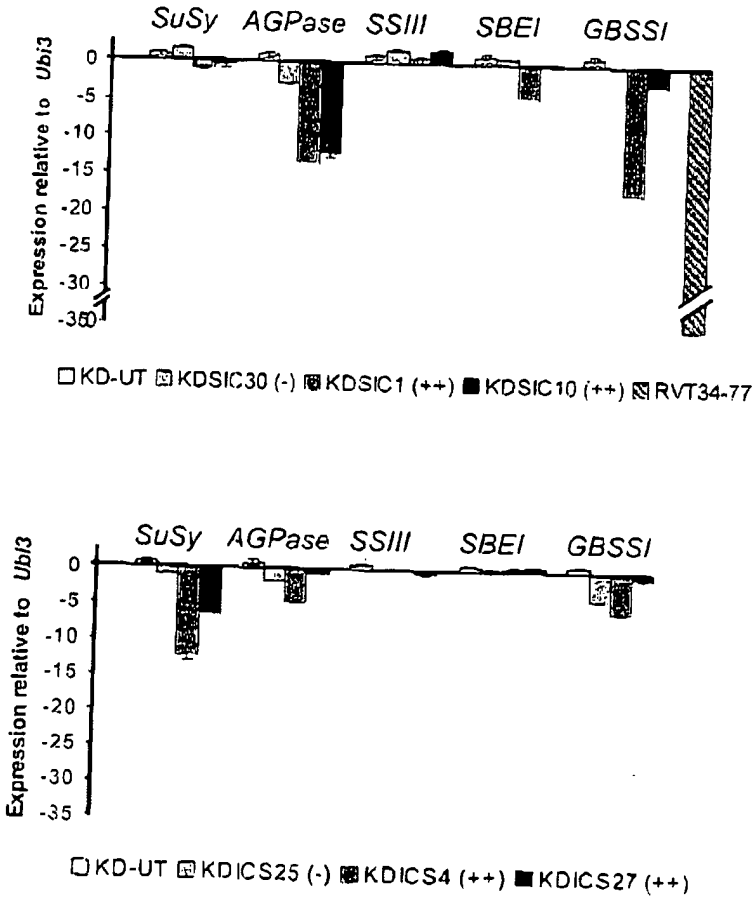

Figure 8. Real-time quantitative RT-PCR analysis of KDSIC30 (–), KDSIC1 (++), KDSIC10 (++), KDICS25 (–), KDICS4 (++), KDICS27 (++) transformants and KD-UT tuber RNA. Expression levels of the following starch synthesizing genes are indicated: *SuSy*, sucrose synthase; *AGPase*, ADP-glucose pyrophosphorylase subunit S; *SSIII*, starch synthase III; *SBEI*, starch branching enzyme I; *GBSSI*, granule-bound starch synthase I. RVT34-77, RNA from Karnico potato tubers expressing a sense/antisense *GBSSI* cDNA construct leading to complete *GBSSI* gene down-regulation. RNA levels for each gene were expressed relative to the amount of *Ubi3* RNA, as described in materials and methods.

in contrast to the KDICS transformants and KD-UT. For *GBSSI*, this down-regulation was 20 times less than for a completely down-regulated *GBSSI* transformant named RVT34-77. Typically, the *SuSy* expression in both high GTFICAT-SBD expressors of the KDICS series was down-regulated, contrary to that in the KDSIC series.

Post-harvest mutan synthesis with GTFICAT-containing starch granules

In an effort to produce more mutan, transgenic starch granules were incubated with an excess of sucrose at 37°C, which is the optimal temperature for GTFICAT, and at 45°C. For this, starch granules from KDSIC10 were used because of their known SBD accumulation inside starch granules, together with the high *GtfICAT* expression in potato tubers. KDIC15, which is a high expressor (Kok-Jacon *et al.*, 2005a), was selected as a control to investigate if granules from this plant can produce mutan post-harvest. Another reason for selecting granules of KDSIC10 and KDIC15 was their spongious appearance (see Fig. 5), which might facilitate the diffusion of sucrose to the granule interior. After 66 h of incubation, the production of fructose and glucose was determined by HPAEC. The release of fructose is indicative for the amount of GTFICAT activity, whereas the release of glucose is not; glucose can either be released as such (hydrolytic activity of the enzyme) or as part of the mutan that is formed (polymerizing activity of the enzyme). From Table 2, it can be seen that GTFICAT was active, but at a low level. After 66 h, fructose was released in a higher amount for the KDIC15 (33 µg/ml at 45°C) transformant than for KDSIC10 (20 µg/ml at 45°C), in contrast to KD-UT for which no fructose was released at all. The glucose concentration was similar to that of fructose, suggesting that the enzyme inside starch granules mainly catalyzes a hydrolysis reaction and no polymerization. This is in line with our observations that no increased amounts of mutan were visualized upon light microscopy analysis of transgenic starch granules, which were stained with erythrosine after 66 h of incubation with sucrose.

Table 2: Summary of post-harvest experiments from KDSIC10, KDIC15 and KD-UT starches by measuring the release of fructose and glucose concentrations in µg/ml by HPAEC at 37°C and 45°C.

Transformants	Fructose (µg/ml)		Glucose (µg/ml)	
	37°C	45°C	37°C	45°C
KD-UT	0	0	0	6
KDSIC10 (++)	10	20	7	16
KDIC15 (++)	13	33	11	28

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Discussion

In this study, a microbial SBD was fused to the N- and C-terminal parts of GTFICAT in order to bring this effector enzyme in more intimate contact with starch granules.

An appended SBD at the N-terminal part of GTFICAT was the most efficient way to produce mutan inside starch granules. Besides its presence on granule surfaces, it was also trapped within the granule matrix, as visualized after exhaustive treatment with exo-mutanase. This result differed from that obtained with GTFICAT alone (Kok-Jacon *et al.*, 2005a), showing that a SBD fused to the N-terminus of GTFICAT can target the enzyme to starch granules. In contrast, SBD fusion to the C-terminus of GTFICAT did not promote the binding of GTFICAT to starch granules and the production of mutan, because the obtained results were very similar to those of untransformed plants. Ji *et al.* (2004b) also indicated that it is difficult to predict the prefered position for SBD in a fusion protein beforehand, and that both the N- and C-terminal position should be investigated.

Several results showed that GTFICAT with an appended SBD had less pronounced effects on starch biosynthesis than GTFICAT alone. (i) Granule morphology was more affected in KDIC starch granules (Fig. 5I and Kok-Jacon *et al.*, 2005a) than in those of KDSIC and KDICS. (ii) The end viscosity of starch pastes was higher for the selected KDIC15 transformant in comparison to that of KDSIC and KDICS transformants (Fig. 7). (iii) Expression of the *AGPase* and *GBSSI* genes were more significantly down-regulated in the KDIC (++) than in the KDSIC (++) and KDICS (++) transformants. This is probably due to a higher sucrose conversion rate with GTFICAT alone, by which the expression level of the sucrose-regulated *AGPase* and *GBSSI* genes is more affected (Geigenberger, 2003; Salehuzzaman *et al.*, 1994). In accordance with these results, the starch content of the tubers from the KDIC (++) transformants was lower than that from the KDSIC (++) and KDICS (++) transformants. (iv) The post-harvest experiments demonstrated that granules from KDIC (++) were more active in presence of excess sucrose than those from KDSIC (++). In itself this was an unexpected observation, since GTFICAT is thought not to have an affinity for the starch granule of its own. However, it is possible that plastidial enzymes are coincidently entrapped in the granule matrix, as has been shown for other "soluble" enzymes such as the potato starch synthase III, that are involved in starch biosynthesis (Marshall *et al.*, 1996).

The less pronounced effects of GTFICAT with appended SBD in comparison with GTFICAT alone might be explained by hypothesizing either that the appended SBD hinders accumulation of fusion protein in the amyloplast, or that the appended SBD reduces the activity of the enzyme. Although the former explanation can not be excluded, we favour the latter, particularly because this is most consistent with the post-harvest experiments; we consider it unlikely that more "soluble" than granule-bound GFTICAT can be accumulated in the starch granule. GTFICAT, which is an enzyme of large molecular weight, might interfere with the proper folding of SBD, and vice versa. Alternatively, the two domains might interact with each other in such a way, that the accessibility of the catalytic site of the enzyme is limited for substrates, or that the aromatic amino acids responsible for the binding of SBD to starch are not exposed anymore (Kok-Jacon *et al.*, 2003). The performance of the fusion proteins might be improved by engineering a different inter-domain linker, which keeps the two domains further apart so that they could function more independently. This remains to be investigated further.

This paper provides the first evidence that starch with different physical properties can be obtained by fusing SBD to an effector (GTFICAT). One of the transformant lines (KDSIC10) produced starch with a 3 °C higher T onset. This increase was very consistent with other observations for this line, including a high transcript level with RT-PCR analysis, detection of SBD by Western blot analysis, the most severely altered granule morphology of the described series, a large amount of mutan in starch preparations, the most deviating viscosity profile, and a large influence on the expression of sucrose-regulated genes as shown by real-time quantitative RT-PCR. Interestingly, these results demonstrate that a covalent attachment of mutan to starch (see debranching analysis) did not seem to be a prerequisite for altering starch properties. The higher T onset of KDSIC10 starch, together with our speculation that GTFICAT alone is probably more active than SBD-GTFICAT, suggests that the site of deposition of the foreign polymer might be more important for altering starch properties than the amount that is actually produced. It is not understood why the T onset of KDSIC10 starch is increased. We think that the presence of mutan would interfere with granule packing, leading to a less ordered structure, and had therefore expected a lower T onset. Since mutan is not well soluble in water, it is possible that the mutan chains interact, forming a co-existing network with starch, which might reinforce the

granule, and increase the melting temperature. It can not be excluded that starch polymers and mutan interact with each other inside the granule. The increased viscosity (retrogradation) upon cooling a paste of KDSIC10 starch compared to KD-UT may indicate this.

This investigation has demonstrated that it is possible to alter starch granule properties by expression of SBD-GTFICAT in potato tubers, the impact of which is larger than with GTFICAT alone. In previous studies, we have shown that more SBD-containing proteins can be accumulated in the *amf* background (Ji *et al.*, 2003, 2004b). Therefore, GTFICAT with an appended SBD has also been introduced in *amf* potatoes, the production of which is in progress. It is expected that the properties of starch derived from these plants are more severely affected than in the Kardal background.

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Microbial starch-binding domain

Romina Rodríguez-Sanoja, Norma Oviedo and Sergio Sánchez

Glucosidic bonds from different non-soluble polysaccharides such as starch, cellulose and xylan are hydrolyzed by amylases, cellulases and xylanases, respectively. These enzymes are produced by microorganisms. They have a modular structure that is composed of a catalytic domain and at least one non-catalytic domain that is involved in polysaccharide binding. Starch-binding modules are present in microbial enzymes that are involved in starch metabolism; these are classified into several different families on the basis of their amino acid sequence similarities. Such binding domains promote attachment to the substrate and increase its concentration at the active site of the enzyme, which allows microorganisms to degrade non-soluble starch. Fold similarities are better conserved than sequences; nevertheless, it is possible to notice two evolutionary clusters of microbial starch-binding domains. These domains have enormous potential as tags for protein immobilization, as well as for the tailoring of enzymes that play a part in polysaccharide metabolism.

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Introduction

The non-catalytic polysaccharide-binding modules are functional domains present in enzymes that are involved in polysaccharide metabolism and in non-catalytic proteins such as lectins and sugar-transport proteins. The first examples of these domains studied were shown to bind to crystalline cellulose, but soon after this a diverse ligand specificity was manifest through the discovery of other natural domains that specifically bind other polysaccharides such as hemicellulose, xylan, chitin, mannan, mutan, lactose, inulin, starch, etc.

Starch-binding domains (SBDs) are present in about 10% of amylases and their related enzymes. They are found in bacterial and fungal α-amylases, bacterial cyclodextrin glucanotransferases, bacterial β-amylases and fungal glucoamylases [1]. All of these enzymes have different catalytic properties and structures, however, all are multidomain proteins that show significant SBD sequence homology. This domain is usually localized at the C-terminal end of enzymes. A few exceptions are the *Rhizopus oryzae* glucoamylase [2], the *Thermoactinomyces vulgaris* α-amylase [3••] and the *Thermotoga maritima* pullulanase [4], which present their SBDs at the N-terminus.

These modules are interesting not only because of their biotechnological potential but also as a model for the study of carbohydrate–protein interactions. This review summarizes the current knowledge of microbial SBD classification, function and structure, particularly focussing on SBD from carbohydrate-binding module (CBM) 20, the best-studied family. Finally, the potential applications of the SBD will be also discussed.

Classification

CBMs were previously classified as cellulose-binding domains (CBDs) on the basis of the initial discovery of several modules that bind cellulose [5,6]. However, additional modules in carbohydrate-active enzymes are continually being found that bind to carbohydrates other than cellulose yet still meet the CBM criteria. Hence, there is a need to reclassify these polypeptides using a more inclusive terminology. On the basis of their amino acid similarity, CBMs have been classified into 42 families [7]. Many of these binding modules have been identified on an experimental basis, but several others have been classified as putative CBMs by primary amino acid sequence similarities.

According to this classification, SBDs belong to six different families of CBMs: CBM20, CBM21, CBM25, CBM26, CBM34 and CBM41. To date, CBM20 is the most generalized family and has been studied in the greatest detail; it contains 120 different proteins and the granular starch-binding function has been demonstrated in several cases. The modules contain ~100 amino acid residues and they might be located after the catalytic domain (CD) or following a long *O*-glycosylated linker region, as seen in fungal glucoamylases [8]. In this family, nine crystalline structures have been determined; some of them were crystallized separately from their CDs. These CDs have diverse catalytic activities, acting as glycoside hydrolases (GHs) and transferases, α- and β-amylases, glucoamylases and pullulanases. The amylases, as the

carbohydrate active enzymes, are classified by their primary structure in several families. CBM20 members have catalytic counterparts within the GH families GH13, GH14 and GH15 [7].

CBM21 contains 56 proteins that are ~100 amino acid residues in length. Even when some of the catalytic counterparts are glucoamylases (GH15) and α-amylases (GH13), most of the proteins included constitute hypothetical proteins that have been derived from genome analysis. The starch-binding function has been demonstrated in only one case. To date, there is no structural information regarding these.

CBM25 has 14 members; their catalytic counterparts are mainly α- and β-amylases from GH13 and GH14. Starch-binding function has been demonstrated in one case [9]. CBM26 includes 18 proteins; the starch-binding function has been demonstrated in two cases [10,11]. The catalytic counterparts are predominantly α-amylases from GH13. There is no structural information for either of these CBM families, and their length and architecture are rather variable. Moreover, some of their members have their SBDs organized in tandem-repeat modules; for example, this is shown in the maltopentaose-forming amylase from an alkaliphilic Gram-positive bacteria DSM 5853 [12], *Bacillus* sp. 195 α-amylase [9], *Clostridium acetobutylicum* ATCC824 α-amylase [13], *Paenibacillus polymyxa* multi-domain amylase [14] and in two *Streptomyces* α-amylases [15,16]. In CBM26, the tandem repeats are present in *Lactobacillus manihotivorans*, *Lactobacillus amylovorus* and *Lactobacillus plantarum* A6 α-amylases [17,18].

82 proteins are present in the CBM34 family, each of which contain ~120 amino acid residues. Granular starch-binding function has been demonstrated in the case of *T. vulgaris* R-47 α-amylase 1 (TVAI) [19]. In this family, five crystal structures have been determined.

CBM41 contains 40 proteins that are composed of ~100 amino acid residues. Members of this family can be found in bacterial pullulanases. The structure of a *Klebsiella* pullulanase has been described [7], but this has not been reported in the protein data bank. The *N*-terminal module of *T. maritima* Pul13 has been shown to bind to α-glucans, amylose, amylopectin and pullulan as well as to oligosaccharide fragments that are derived from these polysaccharides [4].

A schematic view of the typical SBD architectures is shown in Figure 1. For more detailed information about GH families and their CBMs visit the carbohydrate-active enzymes database (CAZY; http://afmb.cnrs-mrs.fr/CAZY/).

Starch-binding domain evolution

The SBD has been identified in α-amylases, β-amylases, maltotetraohydrolases, maltopentaohydrolases, maltogenic α-amylases, cyclodextrin glucanotransferase (CGTase), acarviose transferases and glucoamylases. This domain has been identified in amylolytic microbial enzymes, in particular those of filamentous fungi, Gram-positive bacteria (e.g. Firmicutes), Proteobacteria of the γ-subdivision, actinomycetes and Archaea [20••].

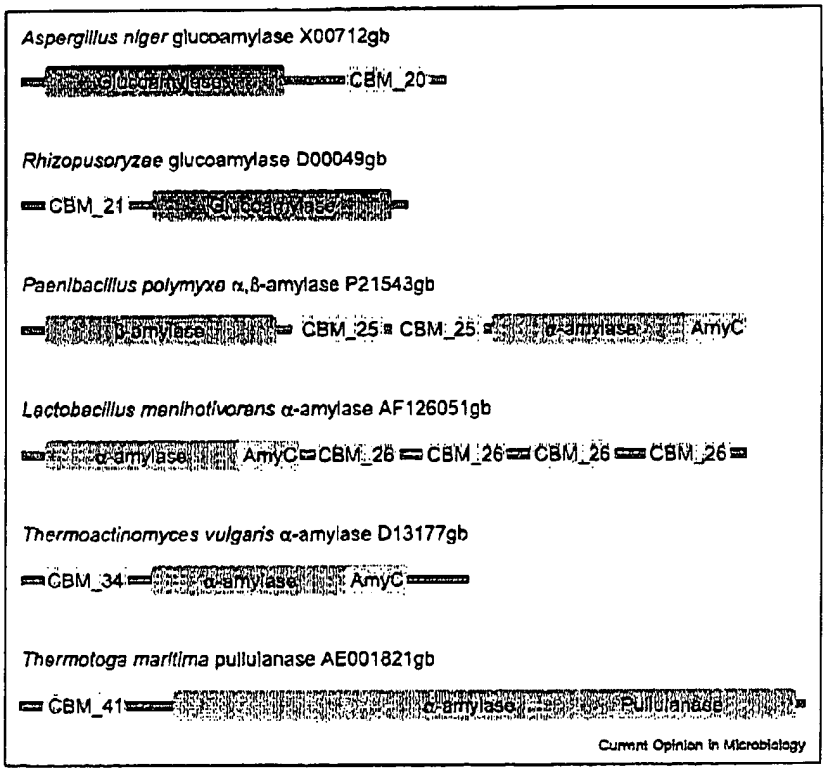
Most amylolytic enzymes belong to three different families of glycosidases: GH13, GH14 and GH15 [21]. Janeček and Ševčík [1] analyzed 42 SBD sequences from the CBM20 family, with the aim of finding an evolutionary relationship between these three glycoside hydrolase families. From their analysis, they postulate that there are two basic groups of SBDs: a bacterial type (that is present in *Bacillus*, *Klebsiella*, *Clostridium* and perhaps *Pseudomonas*) and a fungal type (that is found in mold and yeast). Peculiarly, actinomycetes seem to contain more amino acid residues that are characteristic of the fungal-type SBD sequence.

In a subsequent phylogenetic analysis of 40 sequences from GH13 members, the full-length enzyme sequences were compared with their isolated SBD sequences. Janeček *et al.* found that the SBD sequences reflect their origin rather than the enzyme specificity to which they belong (e.g. α-, β- or gluco-amylase), whereas the core (i.e. catalytic) sequences of the enzymes are clustered according to their function (Figure 2) [20••]. These observations suggest that SBDs have an intragenomic origin and imply that the distribution of the C-terminal domains probably occurred by duplication, which generated the tandem repeats observed in some amylases that have SBDs from families CBM25 and CBM26. Moreover, the widespread distribution of CBM20 in bacteria strongly suggests its bacterial origin [22]. However, two cases of CBM20-like domains have been detected in the mammalian proteins laforin [23] and genethonin [24].

Starch-binding domain structure

Currently, the structures of four SBDs from microbial amylases and CGTases have been reported in the Molecular Modelling Database of the National Center for Biotechnology Information (NCBI) (from *Aspergillus niger*, *Bacillus circulans*, *T. vulgaris* and *Bacillus cereus* var. *mycoides*). The *A. niger* glucoamylase SBD was the first structure to be elucidated and is taken to be the representative SBD (a member of the CBM20 family). This domain consists of eight β-strands that form two major β-sheets: the first β-sheet contains five antiparallel strands and the second includes one parallel strand and one pair of antiparallel strands [25]. The N- and C-termini are located at opposite ends of the molecule. This assembly generates an open-sided distorted β-barrel structure that has a disulphide bond between the N-terminus and the loop formed by the 7th and 8th strands. The three-dimensional structure shows the presence of two substrate-binding sites (Figure 3). [26].

Figure 1



The typical architectures of starch-binding domains (SBDs). The six SBD families are represented (CBM20, CBM21, CBM25, CBM26, CBM34 and CBM41). SBDs are found at the N- or C-terminal of the catalytic domain and are represented with yellow boxes. Glucoamylase catalytic domains (CDs) are identified in red, the β-amylase CD in purple, α-amylase CDs in green and pullulanase CD in beige. Accession numbers are from GenBank.

In addition, crystal structures of the free CGTase and of the CGTase–maltose complex from *B. circulans* and *Bacillus stearothermophilus*, respectively, have been determined [27,28]. CGTases perform the intramolecular transglycosylation of starch into a mixture of α-, β- and γ-ciclodextrins. This CGTase SBD shares almost 37% amino acid sequence identity with the glucoamylase SBD of *A. niger*. Their three-dimensional structures are very similar in their β-sheet characteristics as well as in the position and the length of β-strands; however, the disulphide bond is absent in the CGTase. This condition does not affect substrate binding [29,30].

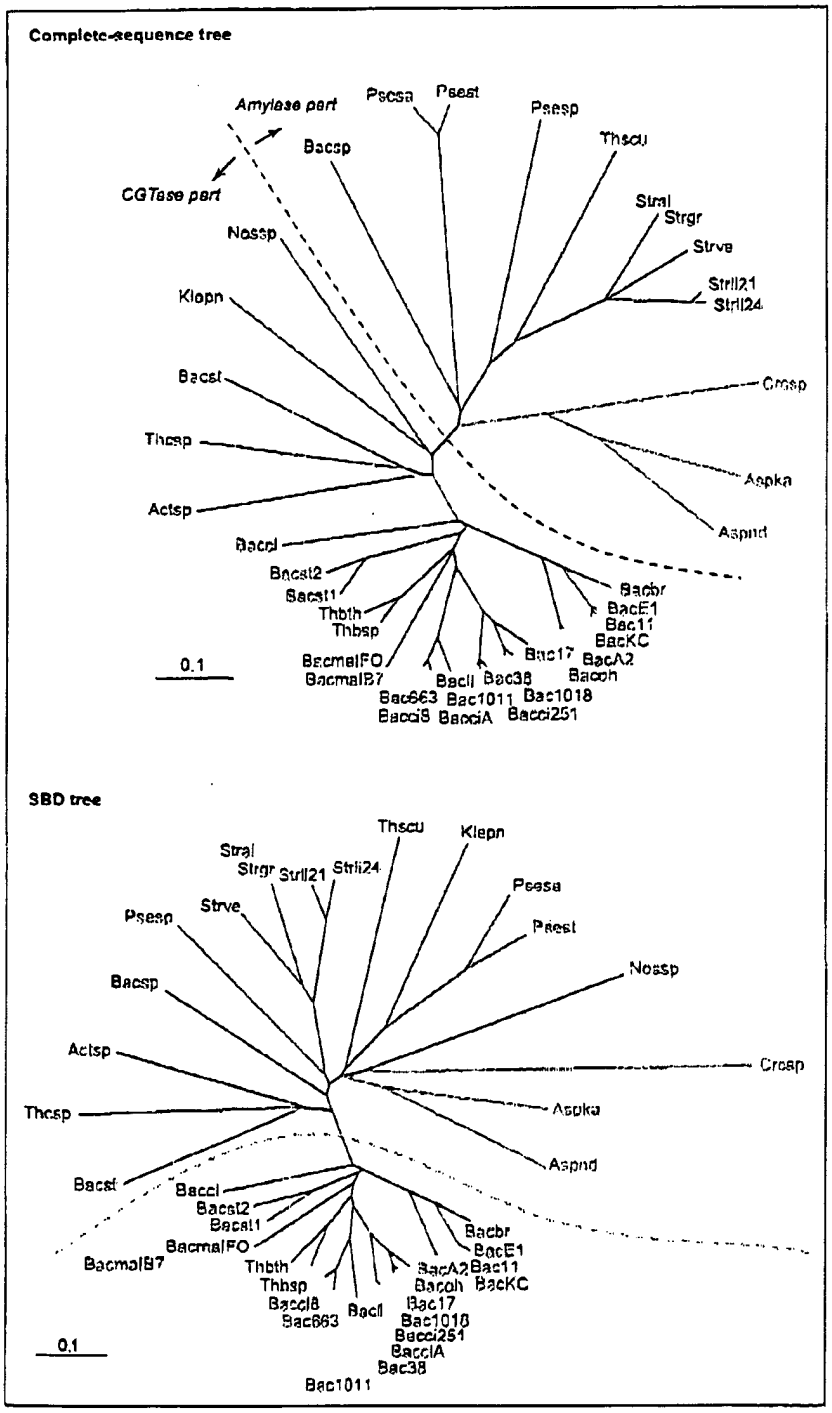
The X ray structures of two α-amylases (TVAI and TVAII) from *T. vulgaris* have been determined. Both showed that the SBD has a distorted β-barrel structure that consists of 121 amino acid residues and that is localized at the N-terminal site. Their structures are similar, but they differ in their orientation. The N domain from TVAI interacts with domains A and C, conferring a spherical structure; however, the N domain of TVAII is isolated from the catalytic domain, forming a dimeric structure that is very suitable for binding small substrates. The interaction between domains N and A of TVAI allows the formation of two binding sites: site N should act as an anchor between the granular starch and the enzyme, whereas site NA is expected to recognize the substrate in a less organized structure [3••].

The three-dimensional structure of *B. cereus* β-amylase C-terminal SBD (BCB-SBD) was compared with the SBDs of *A. niger* glucoamylase and of *B. circulans* CGTase (Figure 3). They have an identity of 22% and 31%, respectively. In spite of this low identity, they share a similar three-dimensional structure — an eight-stranded Greek key topology that has a molecular weight of ~10 kDa. The BCB-SBD is folded into two four-stranded antiparallel β-sheets, which form an eight-stranded antiparallel β-barrel [31].

Starch-binding domain function in binding and hydrolysis

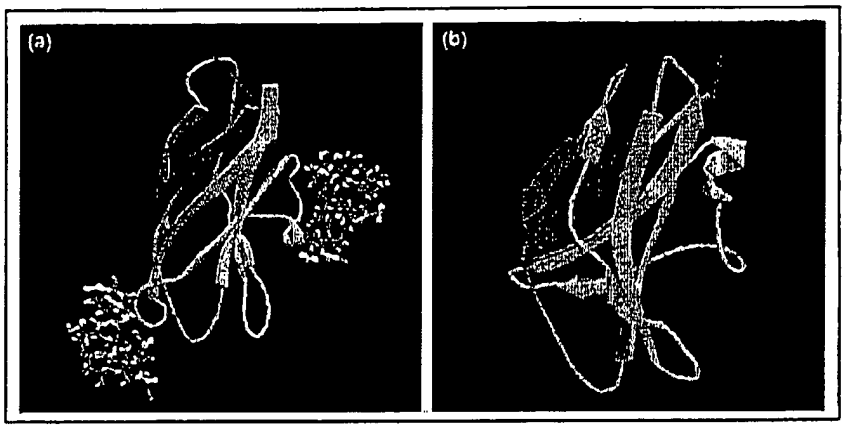
In full-length amylases, the SBD has three known roles: it allows the interaction between the insoluble substrate

Figure 2



The evolutionary trees of the (a) full-length sequences of 40 GH13 members and (b) their isolated SBD sequences. Colour code: red, CGTases; yellow, acarviose transferase; pink, maltogenic α-amylase; blue, α-amylases from Bacillus and actinomycetes; light blue, α-amylases from fungi and yeast; green, maltotetraohydrolases and maltopentaohydrolase. The abbreviations of the analyzed enzymes are shown. For abbreviations and SWISS-PROT accession numbers please refer to [20••]. Reproduced from Janeček *et al.* with permission [20••].

Figure 3



Schematic diagram of the three-dimensional structure of: **(a)** *Aspergillus niger* glucoamylase SBD (protein databank identification (PDBID):1ACO [31]) complexed with cyclodextrin (structures in red/grey/white), the representation shows the two substrate binding sites; and **(b)** *Bacillus cereus* β-amylase SBD (PDBID:1CQY [40]). This domain only has one binding site because of the incomplete generation of the second binding site. Reproduced from the protein data bank (URL: http://www.pdb.org) [46].

and the enzyme in solution; it brings the substrate to the active site in the CD; and in some cases it has a disruptive function on the surface of the starch granule.

In several amylases, enzyme adsorption is a prerequisite for raw starch hydrolysis [10,32,33], perhaps because of the SBD cooperativity that increases the local substrate concentration at the active site of the CD [34,35].

SBD adsorption was initially studied in *Aspergillus awamori* var. *kawachi* glucoamylase. Goto *et al.* [36] found that PL(W-563)YVTVTLPA is the minimal sequence necessary for the digestion of raw starch. The hydrophobic residue W563 contributes to formation of an adsorption complex. The sequence near W590, which has abundant hydrogen bond-forming residues and the charged amino acid residues that are needed for stable SBD adsorption into raw starch, probably assists in the formation of the adsorption complex.

Additionally, Lawson *et al.* [28] found two maltose-binding sites in the CGTase SBD of *B. circulans*. They proposed that the binding of maltose at the first binding site occurs mainly through hydrophobic stacking of both glucose rings from maltose on the indole groups of W616 and W662. In the second site, the reducing glucose unit of maltose is stacked over the phenyl ring of Y633. Modeling studies suggest that a longer amylase chain could be accommodated in an extended ribbon conformation that interacts with both sites.

NMR studies of *A. niger* glucoamylase SBD that is bound to β-cyclodextrin (a cyclic starch analogue) [37] showed that there are two independent binding sites in the SBD that are structurally and, probably, functionally different. The interaction in the first site is compact and is quite rigid, as shown above for SBD–β-cyclodextrin. The major interactions between the SBD and starch involve only two glucose units, which stack over W543 and W590. The second site displays a more extended surface of interaction. The starch molecules in this site span a much larger conformational space. The main protein–carbohydrate contacts involve Y556 of the SBD, which forms a fixed pivot, and Y527, which can swing around in an arc taking the starch strand with it. The starch helices in the two binding sites adopt a markedly nonparallel geometry; the difference in orientation is approximately 90°. This is significant because it is thought that the starch helices on the surface of starch granules are normally parallel — this must be true for the crystalline regions of starch. Two, quite different, functional reasons for the nonparallel geometry are plausible: either the SBD forces two starch strands apart and thus assists in the breakdown of starch granules or the SBD localizes the enzyme to noncrystalline (and therefore presumably to more easily hydrolysable) regions of starch [37,38]. A similar explanation has been postulated for one of the functions of the *Cellulomonas fimi* endoglucanase A CBD [39]; this was later demonstrated experimentally by Southall *et al.* [35]. However, it is unclear whether this is a general mechanism because the two binding sites of the SBDs from the CBM20 family have been shown to be required for amylase-structure disruption [40]. Moreover, it has been reported that the BCB-SBD only binds to one maltose molecule. A comparison between BCB-SBD and the SBDs from *B. circulans* CGTase and *A. niger* glucoamylase reveals some amino acid deletions or insertions that produce an incomplete second binding site, which implies that a conformational change occurs and

explains its inability to bind the second maltose molecule (Figure 3) [31].

Different rates of reaction have been observed for different amylases in the presence and the absence of the SBD, which indicates that SBDs might also play a role in soluble starch hydrolysis [10,9]. Abe *et al.* [3••] suggest that the SBD forms two types of starch-binding sites as the structure of starch is thought to vary from a rigid helical structure in the crystal state of amylose to a loose helical structure when in solution. Consequently, it is expected that the SBD plays a role in helping the enzyme to approach starch by recognizing the surfaces of its relatively rigid helical structures. Also, the SBD is expected to specifically recognize the loose helical structure of starch to help the catalytic site interact with this structure. This is because the rigid helical region of starch cannot bind to the catalytic site of the enzyme.

Biotechnological applications

The main potential applications of SBDs are related to the purification and the immobilization of biologically active proteins — this is of great importance to both industry and research. Starch is an attractive matrix for affinity purification and immobilization, mainly because of its combination of excellent physical properties and low price. To exploit the characteristics of this matrix, investigators have used SBDs from microbial amylases as affinity tags for the purification of recombinant proteins. This strategy can also be used for enzyme immobilization. Using this idea, Dalmia and Nikolov [41] expressed a β-galactosidase–SBD fusion protein in *Escherichia coli*. In this work, the SBD is used as an affinity purification tag and it is shown that after elution from a starch column with maltodextrin the purified fusion protein maintains its β-galactosidase activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels and western blots showed that the purity of the fusion protein eluted from the starch column was as good as, or better than, the protein obtained by conventional affinity chromatography. A similar system has utilized a CBD that is fused to β-glucosidase and is immobilized on cellulose to form a bioreactor. The bioreactor was operated continuously for two weeks at 4–37 °C without loss of activity [42].

Another platform technology, which is widely applicable in starch bioengineering, utilizes SBDs in a different context. SBDs can be used as tools to anchor proteins (that do not have affinity for starch granules) inside starch granules during their biosynthesis. [43]. Modification of starch biosynthesis holds an enormous potential for the production of granules or polymers with new functions. For this purpose, the SBD-encoding region of cyclodextrin glycosyltransferase from *B. circulans* was fused to the sequence that encodes the transit peptide (responsible for amyloplast entry) of potato granule-bound starch synthase I. The synthetic gene is expressed in the tubers of one amylose-free (*amf*) potato mutant and the SBD is accumulated inside starch granules; consequently, the SBDs are protected from protease action. Separate SBDs were targeted to the granule during the starch biosynthetic process. The physicochemical properties of starch (e.g. melting temperature, granule-size distribution and the apparent amylase content) are not affected by the presence of the SBD. An attractive feature of SBD technology is that enzymes introduced in the starch granule can be activated during processing. Thus, reactions that do not normally occur in amyloplasts can be catalyzed. This strategy might be used as an alternative to certain chemical derivatization procedures. In addition, SBD technology provides the opportunity to make starch-based protein carriers. Enzymes or receptors can be immobilized in this way to catalyze particular reactions or for affinity-purification of certain compounds, respectively.

Natural and chemically modified starches are among the most useful biopolymers to be employed in industry. Their application is frequently related to the starch-granule size. This is an important parameter when using starch in the manufacture of biodegradable plastic films. The SBD derived from *B. circulans* cyclodextrin glycosyltransferase was introduced into the amylose-free potato mutant *amf* using appropriate signal sequences [44]. In several transformants, particularly *amf*SS3, the starch granules were much smaller than in control plants (the mean granule size was 7.8 μm compared with 15.2 μm in the control) whereas other starch properties remained unaltered.

An interesting application of SBDs might be to obtain starch with different strengths and to explore their potential in food and textile applications. In the food industry, adhesion of *Bifidobacterium* strains to native maize, potato, oat and barley starch granules has been examined to exploit probiotic food technologies. Starch adhesion was not a characteristic of all 19 bifidobacteria tested. Highly adherent strains were able to hydrolyze the granular starches and adhesion was mediated by a cell surface protein(s) [45]. Adhesion appeared to be specific for α-1,4-linked glucose sugars as it was inhibited by maltose, maltodextrin, amylose and soluble starch but not by trehalose, cellobiose or lactose. Although the cell surface protein of adherent *Bifidobacterium* strains has not been characterized, it can be assumed that SBDs expressed in this way could play a similar role. Therefore, SBDs can be expressed in other non-adherent bifidobacteria to make them susceptible to starch-binding for probiotic applications.

Conclusions

SBDs are essential for raw starch degradation; their presence in microbial amylases must be the result of the

insoluble nature of starch granules. It is interesting that only 10% of the amylases tested seem to have SBDs [1]. This suggests that these domains have been obtained from a recent acquisition, as implied by their isolated phylogeny [20••]. Furthermore, it is important to notice that the screening methodologies and the hydrolytic assays are usually based on the product liberation from soluble substrates without direct measurement of the raw starch-binding. This omission would be compensated for by genome analysis as long as we are able to pass from '*in silico*' analysis to '*in vivo*' studies.

Further studies on the cooperativity between the CD and the SBD, and the SBD structure from others than CBM20 members, are still a requirement for the better understanding of their mechanism of action and for the rational design of specific and effective proteins for raw starch-binding and hydrolysis.

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